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Mapping resistance to the CCR5 co-receptor antagonist vicriviroc using heterologous chimeric HIV-1 envelope genes reveals key determinants in the C2-V5 domain of gp120

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Abstract

Several small molecule drugs that bind to the host CCR5 co-receptor and prevent viral entry have been developed for the treatment of HIV-1 infection. The innate variability found in HIV-1 envelope and the complex viral/cellular interactions during entry makes defining resistance to these inhibitors challenging. Here we found that mapping determinants in the gp160 gene from a primary isolate RU570-VCV$_{res}$, selected in culture for resistance to the CCR5 entry inhibitor vicriviroc, was complicated by inactivity of the cloned envelope gene in pseudovirus assays. We therefore recombined the envelope from RU570-VCV$_{res}$ into a highly active and susceptible ADA gp160 backbone. The chimeric envelopes generated robust signals in the pseudovirus assay and a 200 amino acid fragment, encompassing a C2-V5 region of the RU570-VCV$_{res}$ envelope, was required to confer resistance in both the single-cycle assay and in replicating virus. In contrast, a chimeric envelope that contained only the V3-loop region from this resistant virus was completely susceptible suggesting that the V3-loop changes acquired are context dependent.

Keywords: HIV-1; CCR5 co-receptor; gp160; gp120; Virus entry antagonist; Vicriviroc; Resistance

Introduction

The current standard of care for HIV-1 infection consists of a combination of antiviral drugs that inhibit one or both of the essential viral-encoded enzymes, reverse transcriptase and protease. This treatment strategy has had a major impact on the treatment outcomes for HIV-1 infection; in many cases suppressing HIV-1 viral loads to undetectable levels for many years (Louie and Markowitz, 2002). However, treatment failures develop over time in a majority of patients on long-term therapy and changes to the antiviral regimen are often necessary to maintain viral suppression (del Rio, 2006). When HIV-1 develops resistance to a particular drug, cross-resistance to other approved drugs within the same class of compounds can also occur. This limits the therapeutic options available for those patients who have developed multi-drug resistance to HIV-1. In this regard, two new classes of small molecule antiviral drugs that target viral entry (Tsibris and Kuritzkes, 2007; Westby and van der Ryst, 2005) or the HIV-1 integrase enzyme (Dayam et al., 2007) have recently been developed for HIV treatment.

Maraviroc (UK-427,857) (Dorr et al., 2005; Fatkenheuer et al., 2005) and vicriviroc (SCH-D, SCH-417690) (Gulick et al., 2007; Schurmann et al., 2007; Strizki et al., 2005) are CCR5 co-receptor antagonists that block CCR5-tropic HIV-1 viral entry. Vicriviroc has recently advanced to Phase III human clinical trials in treatment-experienced patients and maraviroc was recently approved by the FDA for use in treatment-experienced patients and is marketed as Selzentry. A more comprehensive review of CCR5 co-receptor inhibitors is described in reviews by Westby and van der Ryst (2005) and Tsibris and Kuritzkes (2007). As these compounds are now being used to treat HIV-1 infection, a clearer understanding for

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how HIV-1 develops resistance to this class of inhibitors has become significantly more important. Currently only limited data are available from *in vitro* resistance studies with different primary isolates (Kuhmann et al., 2004; Marozsan et al., 2005; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007).

In previous studies, *in vitro* generated resistant variants of a sub-type B HIV-1 primary isolate CC1/85 were generated to the CCR5 co-receptor antagonists, AD101 (Kuhmann et al., 2004; Trkola et al., 2002) and vicriviroc (Marozsan et al., 2005; Pugach et al., 2007) in peripheral blood lymphocytes (PBL); each adapting to growth in the presence of very high concentrations of antagonist. Sequence analysis of envelope clones from the resistant variants revealed different patterns of adaptive amino acid changes in the envelope protein as a result of the selective pressure by these different co-receptor antagonists. CC1/85 escape variants to AD101 contained four V3-loop amino acid changes that conferred complete resistance to AD101 (Kuhmann et al., 2004), whereas the CC1/85 virus culture resistant to vicriviroc developed multiple amino acid changes throughout gp160 without any changes in the V3 loop relative to the input isolates (Marozsan et al., 2005). In addition to these studies, *in vitro* generated resistant variants to maraviroc were generated with the same CC1/85 primary isolate and a Clade G RU570 primary isolate (Westby et al., 2007). In this study, two adaptive amino acid changes in V3 loop of the CC1/85 isolate, and a deletion of 3 amino acids within the V3 loop for the RU570 isolate, were primarily responsible for high-level resistance to maraviroc (Westby et al., 2007). These changes were distinct for those reported for AD101 and interestingly these viruses remained susceptible to SCH-C and aplaviroc.

In addition to understanding the underlying mechanism involved in the development of resistance to CCR5 antagonists, concerns still remain as to whether antiviral therapy targeting the CCR5 co-receptor will drive the emergence of CXCR4-tropic viruses during prolonged therapy. These viruses usually appear in the later stages of HIV-1 disease progression as the immune system becomes impaired, and are associated with a rapid acceleration in disease progression (Koot et al., 1999; Philpott, 2003; Shankarappa et al., 1999). However, most of the HIV-1 variants that developed resistance to CCR5 co-receptor antagonists *in vitro* remained CCR5-tropic, with the exception of one resistant variant (Kuhmann et al., 2004; Marozsan et al.,

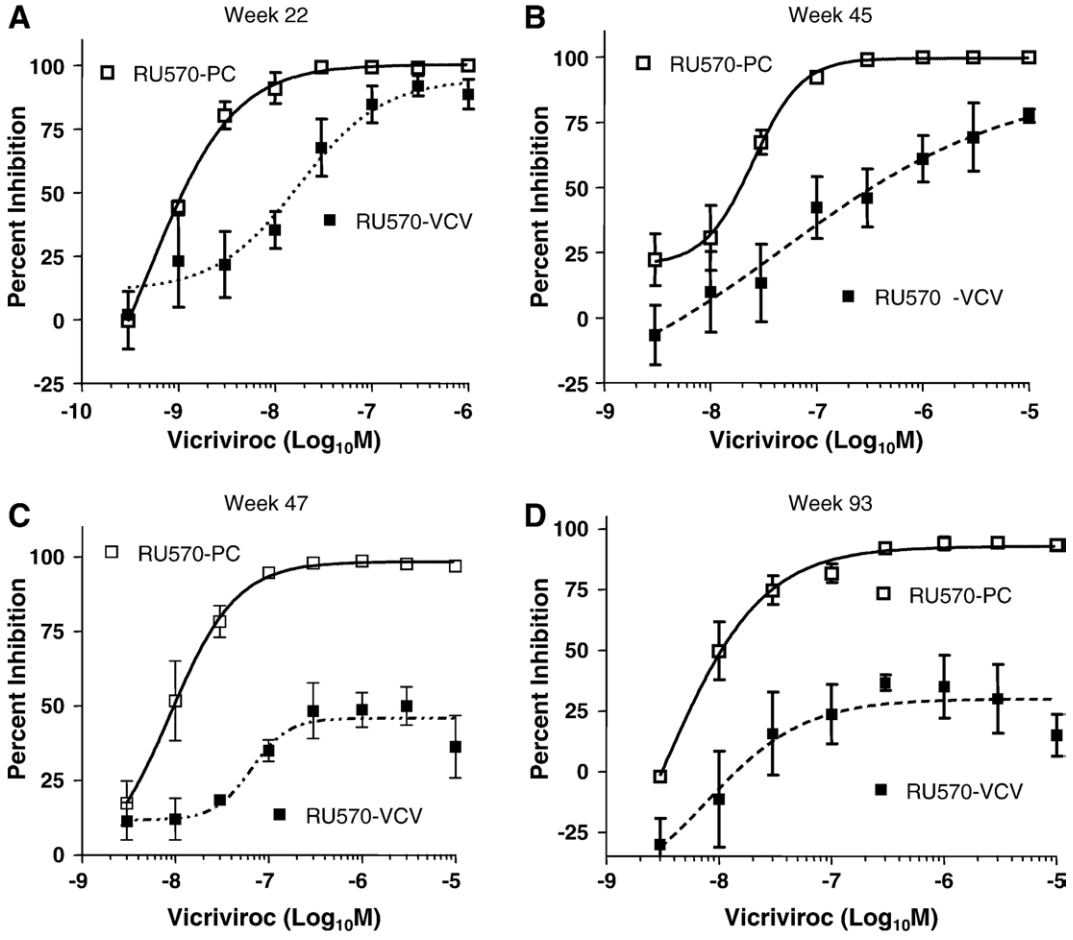

Fig. 1. Vicriviroc susceptibility assays for RU570-PC (-□-) and RU570-VCV (-■-) cultures passaged in PM-1 cells were performed in PBL and dose–response curves representing week 22 (A), week 45 (B), week 47 (C), and week 93 (D) are presented. Curves were generated with PrismGraphPad Software program, Version 4.0 using a nonlinear regression 4-parameter logistic curve fit analysis (R^2 values, RU570-PC: 0.90–0.98, RU570-VCV: 0.80–0.97).

2005; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Although clinical data are limited, accelerated viral evolution toward CXCR4 has not been observed in the on-going CCR5 co-receptor antagonist clinical trials. In a small number of patients treated with maraviroc, CXCR4-tropic virus became detectable over the course of ten days of monotherapy. However, it was shown that these patients had pre-existing CXCR4 tropic virus which fell below the detection limits of a phenotypic assay used to screen patients and did not result from de novo viral mutations (Westby et al., 2006).

In order to better understand HIV-1 resistance to vicriviroc, a vicriviroc-resistant virus culture was generated by passaging a clade G, HIV-1 primary isolate RU570 in PM-1 cells in the presence of increasing concentrations of vicriviroc. This report describes the phenotypic and genotypic properties of these viral cultures using a novel approach to map the genetic determinants of resistance. We used homologous recombination in bacteria to transfer gp120 domains from resistant envelope clones into the background of a heterologous, vicriviroc susceptible envelope and screened for resistance to vicriviroc with these chimeric

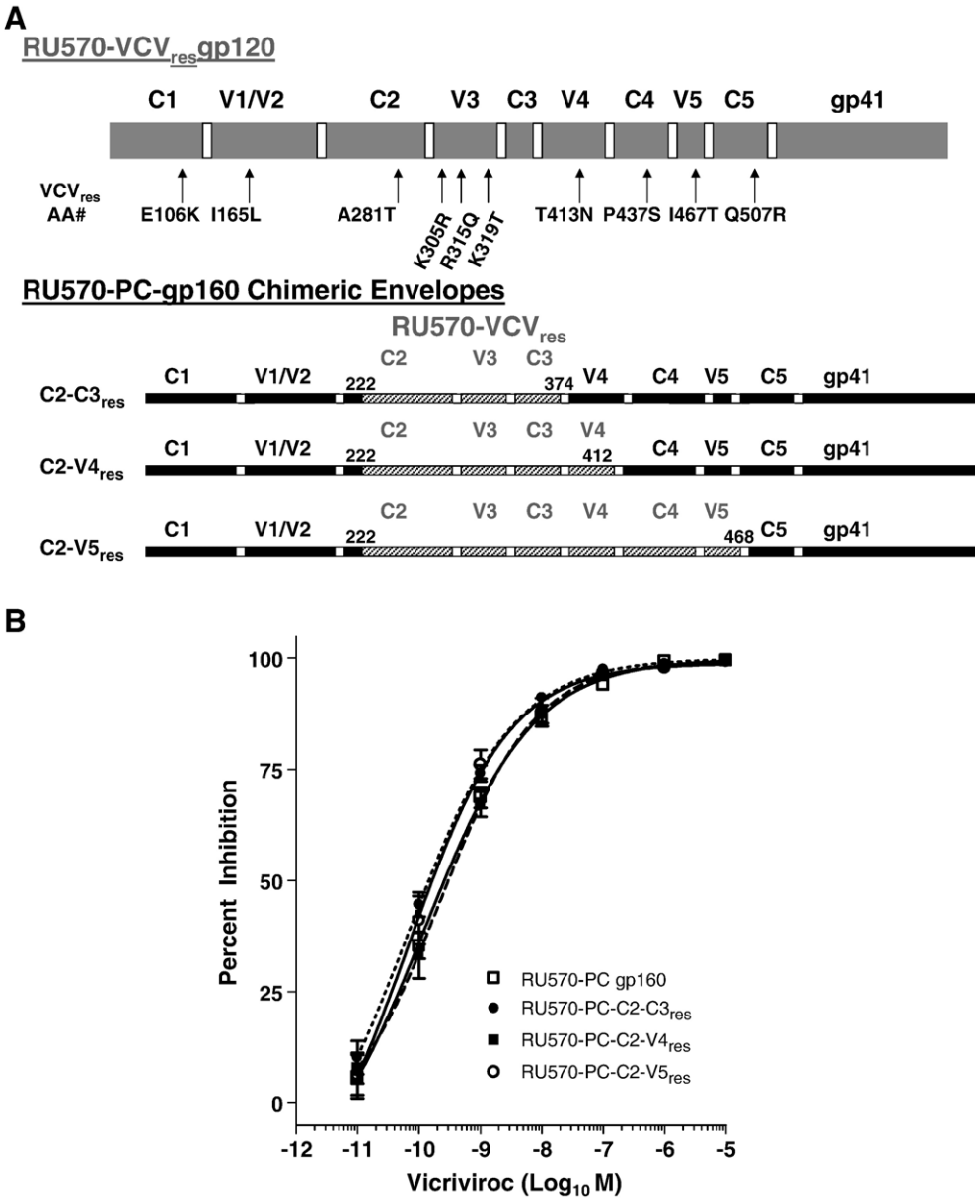

Fig. 2. (A) Schematic of the chimeric envelopes generated by homologous recombination in *BJ5183 E. coli* bacterial cultures. The hatched bars represent the gp120 sequences from VCV$_{res}$ gp120 clones and the filled bars represent the envelope sequence from RU570-PC gp160 into which these sequences were recombined. Amino acid changes that were identified in VCV$_{res}$ gp120 are designated by arrows using HXB2 amino acid coordinates. (B) HIV-1 pseudoviruses generated with VCV$_{res}$ chimeric RU570-PC envelopes were analyzed for vicriviroc susceptibility in U87-CD4-CCR5 cells. Dose–response curves for vicriviroc-susceptibility for each HIV-1 pseudovirus (□—RU570-PC gp160, ●—RU570-PC-C2-C3$_{res}$, ■—RU570-PC-C2-V4$_{res}$, ○—RU570-PC-C2-V5$_{res}$) are represented. Data were analyzed using a nonlinear regression 4-parameter logistic curve-fit analysis with PrismGraphPad Software Version 4.0 (R^2 values, RU570-PC: 0.91, RU570-PC-C2-V5$_{res}$: 0.95).

envelopes in single-cycle HIV-1 pseudovirus assays. In addition, we mapped key resistance mutations identified in the chimeric envelopes by site-directed mutagenesis to determine the effects of these changes on resistance.

Results

Generation of vicriviroc-resistant RU570 HIV-1 variant in PM-1 cells

PM-1 viral cultures were established as described in the Materials and methods section and were assessed periodically for susceptibility to vicriviroc in PBL replication assays between weeks 12–93. Vicriviroc dose–response curves for viruses obtained from weeks 22 (Fig. 1A), 45 (Fig. 1B), 47 (Fig. 1C), and 93 (Fig. 1D) are depicted in Fig. 1. The RU570-VCV virus was completely susceptible to vicriviroc until week 45 showing IC_{50} values <50 nM and maximum percent inhibition equivalent to 100% in dose–response curves similar to that depicted at week 22 (Fig. 2A). At weeks 45 and 47 incomplete dose–response curves to vicriviroc were observed (Figs. 2B and C) with maximal percent inhibition approximately 76% and 45%, respectively. As expected, the RU570 passage control virus remained susceptible to vicriviroc through 93 weeks of cell culture. By week 93, complete resistance in the vicriviroc treated culture was demonstrated by a flat dose–response curve to drug and a maximum percent inhibition at approximately 30% (Fig. 2D).

In order to determine that viral replication in the presence of vicriviroc was not due to co-receptor switching of this primary isolate, we infected U87-CD4 cell lines that express CXCR4 with virus from the week 93 cultures. As has been reported for other *in vitro* generated HIV-1 resistant variants to CCR5 co-receptor antagonists (Kuhmann et al., 2004; Trkola et al., 2002; Westby et al., 2007), the RU570 vicriviroc-resistant variant ($RU570\text{-}VCV_{res}$) from week 93 did not replicate in U87-CD4-CXCR4 cells demonstrating that this resistant variant remained CCR5-tropic only (data not shown).

Clonal sequence analysis of HIV-1 gp120

U87-CD4-CCR5 cells were infected with week 93 cultures for RU570-PC and $RU570\text{-}VCV_{res}$ virus in the absence and presence of vicriviroc, respectively. HIV-1 gp120 sequences were amplified by PCR from genomic DNA isolated from infected cells 48 h post-infection and clonal sequence analysis of gp120 was performed. The amino acid changes in gp120 identified between the RU570-PC and $RU570\text{-}VCV_{res}$ included 10 dominant amino acid changes (present in ≥50% of resistant clones) that were identified, throughout the $RU570\text{-}VCV_{res}$ gp160 protein sequence, following analysis of 18 clones compared with the amino acid sequence of the RU570-PC virus (5 clones). The following dominant amino acid changes were identified: E106K in C1, I165L in V2, A281T in C2, K305R, R315Q, and K319T in the V-3 loop, T413N in V4, P437S in C4, I467T in V5, and Q507R/E in C5 (see Fig. 2). In addition, two amino acid changes in C3 (S363P and T373A), two additional changes in the V3 loop (I317F and G321D), and one amino acid change in C5 (K503R) were found in <50% of the clones.

Generation of chimeric envelopes by homologous recombination in bacteria

Single-cycle assays using pseudotyped HIV-1 particles that express a reporter gene in infected cells are used to address the susceptibility of HIV-1 envelope clones to co-receptor antagonists. A luciferase reporter-gene located in the nef-coding region and a frame-shift mutation in the envelope gene in pNL4-3-$Env^{-}Luc^{+}$ limit the detection of pseudovirus infectivity to a single-round. These assays are also being used to diagnose co-receptor usage for patients entering clinical trials for CCR5 antagonists (Coakley et al., 2005). Unfortunately, efforts to generate pseudoviruses with gp160 clones obtained from $RU570\text{-}VCV_{res}$ cultures using the standard pNL4-3$Env^{-}Luc^{+}$ HIV-1 replication-incompetent vector resulted in pseudovirus stocks with no detectable luciferase activity. In addition, a majority of the gp160 clones from RU570 passage control (PC) virus failed to generate viable pseudotyped HIV-1 particles. Following screening of numerous gp160 clones, we eventually identified a single functional gp160 clone that could be pseudotyped with the HIV-1 vector (Fig. 3).

Therefore, we used an alternate strategy to generate functional envelope clones from resistant viruses. Making use of homologous recombination in bacteria, we cloned different

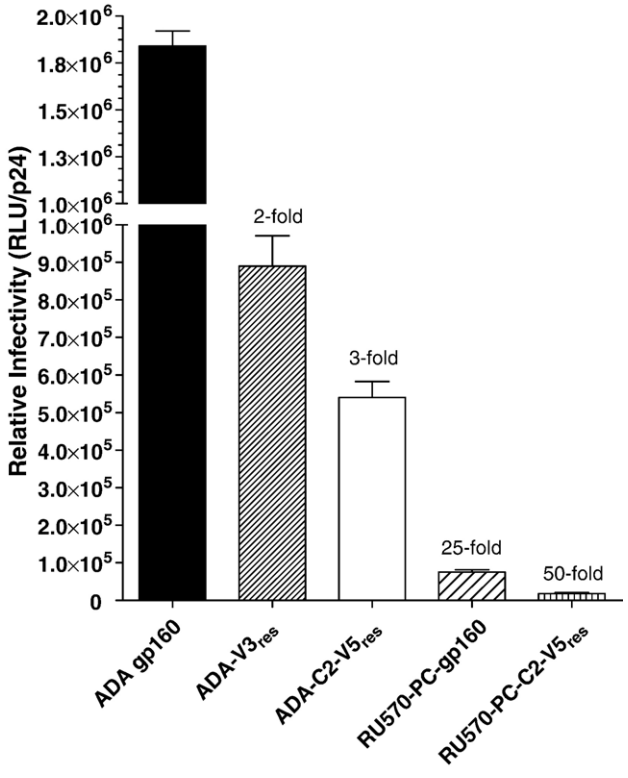

Fig. 3. The relative infectivity (RLU/p24) of HIV-1 pseudoviruses generated with chimeric envelopes was compared to ADA gp160 and RU570-PC gp160 pseudoviruses. Data represent the luciferase activity (RLU) in cells 72 h post-infection that is normalized to the p24 inoculum.

gp120 domains from the RU570-VCV$_{res}$ clones into the background of the RU570-PC gp160 backbone in order to determine if domain swapping would result in the transfer of the resistance phenotype into this vicriviroc-susceptible passage control envelope. Based on numerous studies using restriction-site fragment swapping for mapping co-receptor determinants within gp120 (Cho et al., 1998; Choe et al., 1996; Cocchi et al., 1996), we reasoned that resistance might also tract with domain swapping. Following homologous recombination of VCV$_{res}$ gp120 into the RU570-PC gp160 construct, the chimeric envelope clones were sequenced to determine the co-ordinates of recombination and were then used to generate HIV-1 pseudotyped particles. Fig. 2A depicts the 3 homologous recombinant envelopes that were obtained in the background of RU570-PC gp160. Vicriviroc dose–response experiments in U87-CD4-CCR5 cells were performed using HIV-1 particles pseudotyped with the chimeric envelopes. As expected, the control RU570-PC envelope was completely susceptible to vicriviroc with an IC$_{50}$ value of 0.1 nM and a 100% maximum inhibition. Surprisingly none of the chimeric envelopes containing varying lengths of C2-V5 VCV$_{res}$ gp120 were resistant to vicriviroc. These chimeric pseudotyped HIV-1 particles displayed maximum inhibition levels of 100% at concentrations of vicriviroc $\geq$100 nM and the IC$_{50}$ values calculated from dose–response curves depicted in Fig. 2B ranged between 0.07 and 0.2 nM. Since we routinely analyze the infectivity of pseudoviruses by comparing the ratio of the luciferase activity (RLU) produced per ng of p24 inoculum for each virus (relative infectivity), we

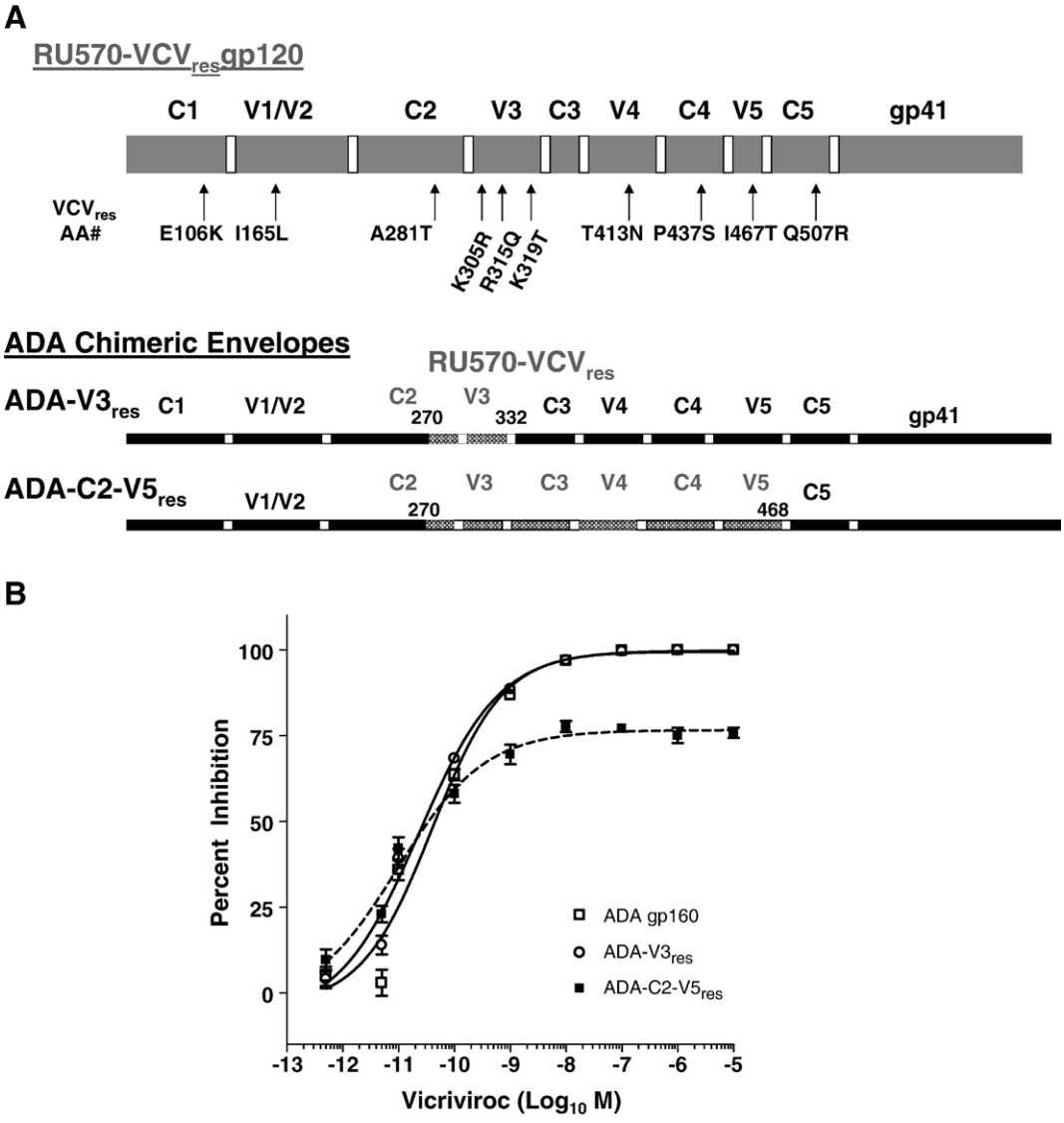

Fig. 4. (A) Schematic of the chimeric envelopes generated by homologous recombination in *BJ5183 E. coli* bacterial cultures. The hatched bars represent the gp120 sequences from VCV$_{res}$ gp120 clones and the filled bars represent the envelope sequence from ADA gp160 into which these sequences were recombined. Amino acid changes that were identified in VCV$_{res}$ gp120 are designated by arrows using HXB2 amino acid coordinates. (B) Dose–response curves for vicriviroc-susceptibility assays were performed for each HIV-1 pseudovirus (□—ADA, ○—ADA-V3$_{res}$, ■—ADA-C2-V5$_{res}$). Data were analyzed using a nonlinear regression 4-parameter logistic curve-fit analysis with PrismGraphPad Software Version 4.0 (R^2 values, ADA: 0.94, ADA-V3$_{res}$: 0.96, ADA-C2-V5$_{res}$: 0.87).

found that the RU570-PC and chimeric envelopes had significantly reduced relative infectivity (25-fold to 50-fold lower) in comparison with HIV-1 particles pseudotyped with ADA envelope (Fig. 3). Since the pseudotyped ADA envelope produced such robust activity, we tried homologous recombination of VCV_{res} gp120 with this completely heterologous, vicriviroc susceptible envelope. Two recombinants containing different fragment lengths of RU570-VCV_{res} gp120 in ADA gp160 (ADA-$V3_{res}$; HXB2 coordinates: aa 270–332; ADA-C2-$V5_{res}$; HXB2 coordinates: aa 270–468) were generated (Fig. 4A) and characterized for infectivity and susceptibility to vicriviroc in single-cycle HIV-1 pseudovirus assays.

Characterization of HIV-1 pseudoviruses containing ADA chimeric envelopes

The luciferase activity for pseudoviruses generated with the ADA chimeric envelopes was enhanced significantly relative to the RU570-PC chimera although it was about 2- to 3-fold lower than wt ADA envelope (Fig. 3). We next performed vicriviroc susceptibility assays using the pseudoviruses generated with the ADA chimeric envelopes. Assays were conducted in U87-CD4-CCR5 cells using serial 10-fold dilutions of vicriviroc spanning a 7–8 $\log_{10}$ molar range in inhibitor concentration and vicriviroc susceptibility curves for these pseudoviruses are shown in Fig. 4B. As expected, control HIV-1 particles pseudotyped with ADA gp160 were completely susceptible to vicriviroc with an IC_{50} value of 0.04 nM and a maximum response of 100% inhibition. Interestingly, pseudoviruses generated with the ADA-$V3_{res}$ chimeric envelope containing only the V-3 loop region from RU570-VCV_{res} gp120 were completely susceptible to vicriviroc despite containing 3 dominant mutations in V-3; whereas pseudoviruses containing the ADA-C2-$V5_{res}$ chimeric envelope exhibited a resistant phenotype showing a reduction in the maximum inhibition (MI) in vicriviroc dose–response curves. This resistant phenotype has been previously reported for other *in vitro* generated resistant variants (Pugach et al., 2007; Westby et al., 2007). These data suggest that the additional C3-V4-C4-V5 domains of gp120 are necessary for maintaining vicriviroc resistance in the background of ADA gp160. Since the V3-loop region from RU570-VCV_{res} gp120 within a heterologous background did not recapitulate resistance, these V3-loop changes are most likely context dependent.

Effect of viral inoculum on maximum inhibition values

Since the ADA-C2-$V5_{res}$ chimeric envelope displayed a vicriviroc-resistance phenotype and the activity was 25-fold higher compared to the RU570-PC-C2-$V5_{res}$ chimeric, we tested increasing doses of virus inoculum with the RU570 chimeric to see if this enabled the detection of resistance for this envelope. Because of the limited infectivity of this chimeric envelope (Fig. 5), we were only able to examine a 10-fold range in p24 inocula. Even with very high amounts of inoculum (90 ng p24), no change in the 100% maximum inhibition occurred. Intriguingly, when the ADA-C2-$V5_{res}$ pseudovirus infections were examined using increasing amounts of virus inocula, the MI for vicriviroc decreased as the level of virus increased (Fig. 5). At high virus inocula (70 ng p24), the plateau level was approximately 55%, however the plateau level increased with decreasing concentrations of virus to approximately 80% MI (2 ng p24). This effect was not seen for HIV-1 pseudoviruses generated with the parental ADA gp160 or RU570-PC gp160 constructs. Using a similar range in virus inocula, the MI values for these viruses remained at 100% regardless of the virus concentration (data not shown).

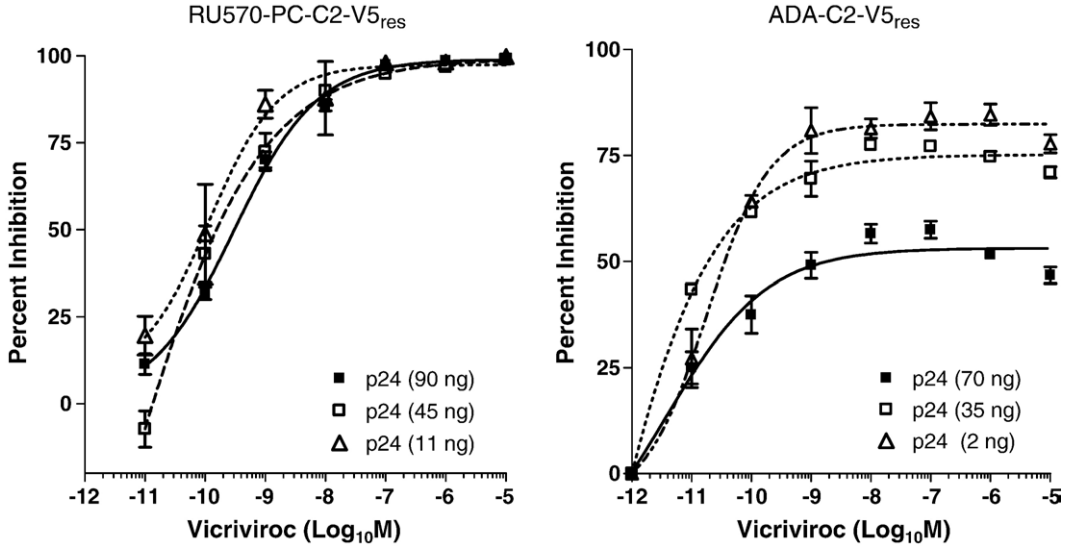

Fig. 5. Variability in the percent maximal inhibition (MI) in single-cycle HIV-1 pseudovirus assays indicative of resistance to vicriviroc correlates with the amount of HIV-1 pseudovirus input based on p24 inoculum for ADA-C2-$V5_{res}$ pseudoviruses. Data were analyzed using a nonlinear regression 4-parameter logistic curve-fit analysis with PrismGraphPad Software Version 4.0 (■—70 ng, □—35 ng, △—2 ng, and R^2 values 0.8, 0.9, 0.95, and 0.8 respectively), whereas no change in MI with the RU570-PC-C2-$V5_{res}$ pseudoviruses was observed (■—90 ng, □—45 ng, △—11 ng and R^2 values 0.99, 0.97, 0.96, and 0.86, respectively).

Site-directed mutagenesis of gp120 amino acids associated with vicriviroc resistance

To determine if resistance to vicriviroc is dependent upon amino acid changes within individual domains of gp120 or on the collective effect of changes throughout gp120, site-directed mutagenesis of six dominant and two nondominant amino acid changes present in the ADA-C2-V5$_{res}$ chimeric envelope gene was performed. Single reverse amino acid changes were made in ADA-C2-V5$_{res}$ at these eight positions by substituting these amino acids with the corresponding amino acid present in the passage control gp120 sequence. These mutations were analyzed for vicriviroc susceptibility in a single-cycle HIV-1 pseudovirus assay as described previously. Because of the dose-dependent effect of viral inocula on MI, our mapping studies were performed with at least four dilutions of pseudovirus spanning the same range in p24 inoculum as ADA-C2-V5$_{res}$ pseudovirus. The results were normalized to virus input (RLU/ng p24) and therefore each data point represents the average of triplicate measurements for each dilution ($n=12$). Data for individual reverse amino acid substitution have been grouped according to their domain location within gp120: V3 loop (Fig. 6A), C3 (Fig. 6B), and V4-C4-V5 (Fig. 6C).

Of the three dominant amino acid changes found in the RU570-VCV$_{res}$ gp120 V3 loop, the reverse mutation having the most dramatic effect on resistance was the R305K substitution in the N-terminal stem-region of the V3 loop. This amino acid substitution resulted in an increase in the maximum inhibition by vicriviroc from 70% to approximately 90% (Fig. 6A; Table 1). These results are similar to those of Westby et al. (2007) demonstrating increases in the MI for maraviroc-resistant viruses following reverse substitution of single, and multiple amino acids in the V3 loop that were acquired during *in vitro* generated resistance. In contrast, the Q315R substitution in the V3 loop had no effect on vicriviroc resistance as demonstrated by the overlapping dose–response curves with ADA-C2-V5$_{res}$ displayed in Fig. 6A. Likewise, combining the two V3-loop reverse substitutions did not produce a cumulative effect and the MI was similar to the single R305K substitution. When the amino acid at position 319 in the crown region of the V3 loop was changed (T319K), no functional pseudoviruses were detected. In order to determine if the amino acid at position 319 was V3-loop context dependent, this amino acid substitution was combined with the R305K and Q315R amino acid substitutions. Again, no functional HIV-1 pseudoviruses were observed. Therefore, the effect of this amino acid on resistance could not be determined in a single-cycle assay.

Single amino acid substitutions made in C3, V4, and V5 had a lesser effect on the maximal percent inhibition. For each single amino acid substitution made, the average plateau level was lower than 80% inhibition and the change in the MI compared to ADA-C2-V5$_{res}$ was ≤10% (Table 1). The S437P amino acid

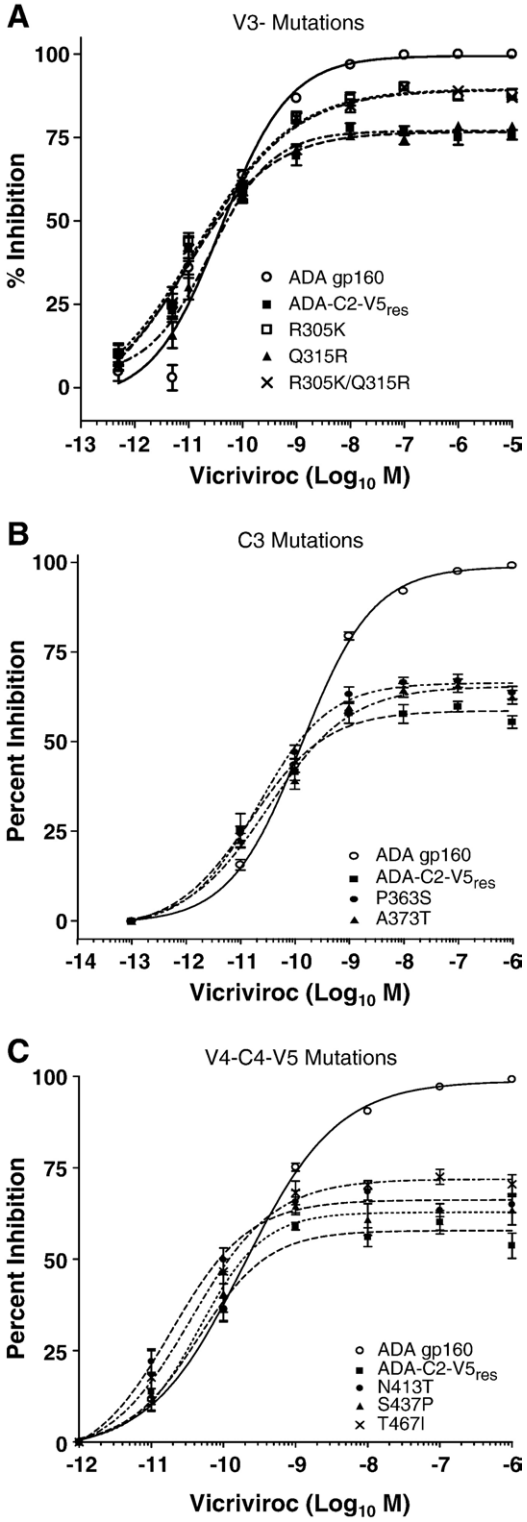

Fig. 6. Dose–response curves for vicriviroc susceptibility assays performed with HIV-1 pseudoviruses generated with chimeric envelope ADA-C2-V5$_{res}$ that contain reverse amino-acid substitutions in gp120 for amino-acid changes identified in PM-1 vicriviroc-resistant RU570 virus cultures compared to RU570-PC. Data were analyzed using a nonlinear regression 4-parameter logistic curve-fit analysis with PrismGraphPad Software Version 4.0. (A) V3-loop mutations; ○—ADA env, ■—ADA-C2-V5$_{res}$, □—R305K, ▲—Q315R, ×—R305K/Q315R, and R^2 values 0.91, 0.87, 0.91, 0.92, and 0.90, respectively. (B) C3 mutations; ○—ADA env, ■—ADA-C2-V5$_{res}$, ●—P363S, ▲—A373T, and R^2 values 0.99, 0.87, 0.91, 0.92, and 0.90, respectively. (C) V4-C4-V5 mutations; ○—ADA env, ■—ADA-C2-V5$_{res}$, ●—N413T, ▲—S437P, ×—T467I, and R^2 values 0.99, 0.92, 0.87, 0.96, 0.83, and 0.96, respectively.

Table 1
Effects of reverse amino acid changes in RU570 vicriviroc-resistant gp120 on susceptibility to vicriviroc in a single-cycle HIV-1 pseudovirus assay

gp120 aa #[a]	gp120 domain	Maximum inhibition (MI)[b] (%)	ΔMI[c] (%)
R305K	V3	89.5±4.9[d]	+19.0±4.0
Q315R	V3	70.7±7.0	+0.2±2.3
T319K[e]	V3	NA	
R305K/Q315R	V3	89.3[d]	+18.8
R305K/P363S	V3/C3	86.9[d]	+16.4
R305K/A373T	V3/C3	86.4±4.3[d]	+16.1±4.1
R305K/A373T/T467I	V3/C3/V5	89.9[d]	+19.4
P363S	C3	74.3	+3.8
A373T	C3	73.2	+2.7
N413T	V4	70.0	−0.5
S437P	C4	65.1	−4.9
T467I	V5	75.6	+5.1
ADA-C2-V5$_{res}$		70.5±7.0	
RU570-PC		100.0	

[a] Amino acid # represents HXB2 gp120 sequence.
[b] Values for ADA-C2-V5$_{res}$ represent the average±standard deviation for $n=5$ independent experiments; R305K, Q315R, R305K/A373T represent $n=3$ and all other values represent the average of at least two independent experiments. Each experiment represents dose–response data for at least four dilutions of pseudovirus.
[c] ΔMI represents the change in the average maximum percent inhibition for each mutation compared to the average maximum percent inhibition for ADA-C2-V5$_{res}$ gp160; (+) ΔMI value correlates to an increased susceptibility to vicriviroc.
[d] $P<0.05$ compared to ADA-C2-V5$_{res}$.
[e] Loss of infectivity in a single-cycle assay; NA, not applicable.

substitution in the C4 domain of gp120 had no effect on the MI. When amino acid substitutions from C3 only or C3 and V5 were combined with the dominant R305K V3-loop substitution, the average MI observed did not increase beyond 90% and was similar to the single R305K substitution as shown by the nearly overlapping dose–response curves in Fig. 7.

Vicriviroc susceptibility assays with replication competent chimeric HIV-1 viruses

CCR5 inhibitor resistance was previously reported to be influenced by the assay format in which resistance is evaluated (Pugach et al., 2007). In assays performed in PBMCs, it was noted that differences were found in the phenotype of a resistant virus displaying noncompetitive resistance in a single-cycle format (plateau effect) versus replication enhancement in a multi-cycle assay. In order to evaluate differences in susceptibility profiles between single-cycle and multi-cycle infection assays in U87-CD4-CCR5 cells, we generated replication competent viruses expressing the chimeric gp120 envelopes. Restriction fragments corresponding to HXB2 aa 203–608 within ADA-V3$_{res}$ and ADA-C2-V5$_{res}$ chimeric envelopes were transferred to an NL4-3-AD8 HIV-1 molecular clone by unique restriction-site digestion and religation. Infectious virus generated in 293T cells was used to infect U87-CD4-CCR5 cells in the presence of increasing concentrations of vicriviroc. Dose–

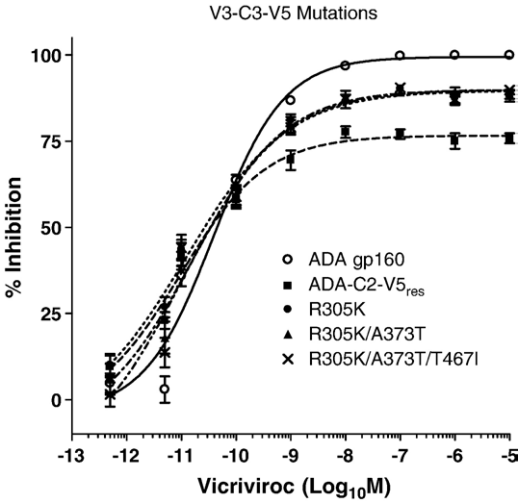

Fig. 7. Dose–response curves for vicriviroc susceptibility assays performed with HIV-1 pseudoviruses generated with chimeric envelope ADA-C2-V5$_{res}$ containing multiple reverse amino-acid substitutions in gp120. Data were analyzed using a nonlinear regression 4-parameter logistic curve-fit analysis with PrismGraphPad Software Version 4.0 (○—ADA env, ■—ADA-C2-V5$_{res}$, ●—R305K, ▲—R305K/A373T, ×—R305K/A373T/T467I, and R^2 values 0.91, 0.87, 0.91, 0.88, and 0.89, respectively).

response data for these viruses are represented in Fig. 8. The replication competent HIV-1 virus containing the ADA-C2-V5$_{res}$ chimeric envelope sequence was resistant to vicriviroc as demonstrated by the incomplete dose–response curve to vicriviroc displaying a MI at approximately 50% in U87-CD4-CCR5 cells. This resistant virus appears to exhibit the same resistance phenotype (plateau effect) in both assay formats. Since the previously published results were performed in PBMCs which would contain secreted chemokines, our results are not directly comparable to those of Pugach et al. because of the differences in experimental conditions. Both NL4-3-AD8 and the NL4-3-AD8-V3$_{res}$ virus containing just the V3-loop region from the resistant gp120 clone were completely susceptible to vicriviroc ($IC_{50}=0.025$ nM).

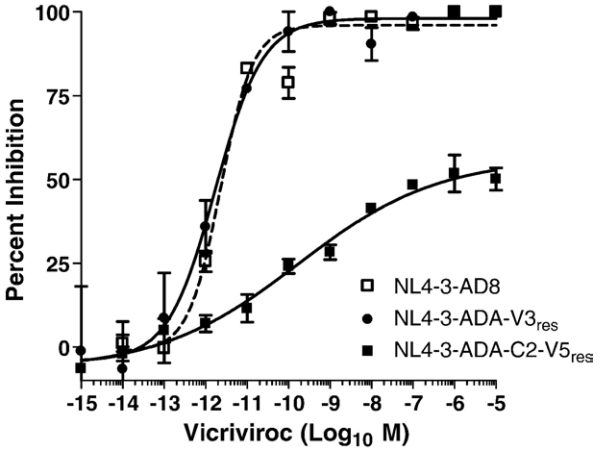

Fig. 8. Vicriviroc dose–response curves for replicating HIV-1 NL4-3-AD8 (-□-), and HIV-NL4-3-AD8 containing the C2-V5$_{res}$ (-■-) and V3$_{res}$ (-●-) gp120 domains from RU570-VCV$_{res}$ gp120 in U87-CD4-CCR5 cells.

Discussion

Single-cycle pseudovirus assays are routinely used to characterize envelope genes from viruses resistant to entry inhibitors. This assay format enables measurement of the efficiency of viral entry for multiple envelopes in a common HIV-1 backbone. In addition, pseudovirus assays have been adapted for high-throughput analysis of clinical samples (Whitcomb et al., 2007). However, the activity level of pseudovirus particles can vary widely for envelope genes cloned from cell-culture generated resistant strains (Westby et al., 2007) or clinical samples. In the present study, we found that pseudoviruses generated with cloned gp160 genes from RU570-VCV_{res} did not produce a measurable signal after infection, and therefore could not be used for characterization of the RU570-VCV_{res} envelope. To overcome this problem, we tried chimeric gp160 envelopes generated with both homologous and heterologous backbones in an effort to increase pseudovirus infectivity. Heterologous chimeric envelopes, generated between the ADA and RU570-VCV_{res} envelope genes, were found to produce robust signals in the pseudovirus assay and were utilized for further characterization of the genotypic and phenotypic determinants of resistance for RU570-VCV_{res}.

Our results demonstrate that resistance to a co-receptor antagonist for an HIV-1 primary isolate can be predominantly contained within a 200-amino acid gp120 domain when transferred into a heterologous, susceptible envelope clone. While domain swapping with gp120 was done for resistance mapping studies with the AD101-resistant CC1/85 chimeric molecular clones, it was into the background of the parental isolate (Kuhmann et al., 2004). In that regard, the gp120 domain (aa 271–386) that showed resistance following restriction-site digestion and re-ligation overlaps with the same region that we identified but ends at the beginning of V4. Therefore, it was surprising that our chimeric envelopes generated in the background of the RU570-PC envelope did not display a resistance phenotype in a single-cycle assay. Based on the lower infectivity observed in pseudovirus assays with these chimeras, it is possible that these envelopes have an overall lower receptor binding affinity. Detection of resistance likely requires a threshold level of envelope affinity that is not achieved with the resistant envelopes in the single round pseudovirus infection assay. Alternatively, it is possible that the rearrangement of gp120 upon binding CD4 and subsequently CCR5 leading to disassembly of gp120/gp41 and triggering of the fusion peptide in the N-terminus of gp41 may be more efficient in the ADA chimera.

Using the heterologous chimeric envelope from ADA-C2-$V5_{res}$, we used site-directed mutagenesis to revert amino acid changes identified within this C2-V5 domain back to the amino acids found in the passage control virus. We found that a single amino acid substitution at position 305 in the V3 loop had the most significant impact on resistance. In our studies, restoration of the 305K residue identified in the passage control sequence into the V3 loop of the resistant envelope conferred partial susceptibility to vicriviroc. Interestingly, this same amino acid change also occurred in the V3 loop of the AD101-resistant virus CC1/85 (Kuhmann et al., 2004) and has also been identified in several patients who experienced viral breakthrough in a phase II clinical study that combined vicriviroc with an optimized background antiviral regimen (Strizki et al., 2006). Recently, Westby et al. described a maraviroc-resistant virus RU570 that contained a deletion of amino acids 315–317 in the V3 crown that conferred resistance to MVC. This pattern of amino acid changes is quite different from the RU570-VCV_{res} V3-loop changes we observed; however, an identical amino acid change occurred at position 319 in both resistant variants. In our studies, when this T319K reverse amino acid change was made in the V3 crown, a complete loss of infectivity occurred in both HIV-1 pseudoviruses and replicating virus. Several reports have identified that position 319 in the V3 crown can contribute to both variations in sensitivity to entry inhibitors and replication fitness (Lobritz et al., 2007; Mosier et al., 1999; Safarian et al., 2006; Torre et al., 2000). In addition to the V3-loop changes, when single amino acid changes were made to individual residues outside the V3 loop, less significant changes in the MI plateau height were observed. Also, several combinations of amino acids outside the V3 loop in conjunction with the dominant R305K substitution did not increase the MI further than the single R305K substitution. These data suggest that a collective contribution of a majority of the amino acids outside the V3 loop in conjunction with the R305K substitution may be needed to restore complete susceptibility to vicriviroc. However, we cannot rule out whether position 319 has an effect on resistance.

The region of gp120 from RU570-VCV_{res}, encompassed in ADA-C2-$V5_{res}$, is well known for its interactions with the CCR5 co-receptor. Results from numerous studies have demonstrated that the V3 crown interacts with CCR5 primarily through the second extracellular loop, and that the V3 base region together with C4 residues in the bridging sheet forms a binding pocket that engages sulfated-tyrosine residues in the N-terminus of CCR5 (Cormier and Dragic, 2002; Hartley et al., 2005; Huang et al., 2007, 2005; Liu et al., 2003; Napier et al., 2007; Rizzuto et al., 1998). Our results suggest that both the V3-loop and C4 regions from RU570-VCV_{res} were necessary to confer vicriviroc resistance in ADA-C2-$V5_{res}$. The ADA-$V3_{res}$ chimera, containing only the V3-loop from RU570-VCV_{res}, was readily able to use the CCR5 receptor in the absence of vicriviroc, but was not able to enter via the drug-bound form. Thus RU570-VCV_{res} may require the unique structural features of both the V3-loop and C4 domain of the resistant viral envelope to interact with the vicriviroc-bound receptor. The importance of the C4 region from RU570-VCV_{res} suggests that the interaction with the N-terminus of CCR5 could be especially important for the development of vicriviroc resistance. Interestingly, a V3 crown deleted HIV-1 R3A strain, TA1, which developed dependence on the N-terminus of CCR5 for viral entry during serial passage in culture, was found to be resistant to CCR5 entry antagonists (Laakso et al., 2007). It is possible that RU570-VCV_{res} may also be more dependent on the N-terminus of CCR5 for viral entry in the presence of vicriviroc. Future studies utilizing specific CCR5 mutant receptors will be required to validate this hypothesis.

Resistance measurements for HIV-1 pseudoviruses generated with the ADA-C2-$V5_{res}$ chimeric envelope gene in single-cycle assays did not result in shifts in vicriviroc IC_{50} values. Rather, the observed phenotype manifested as a reduction in the MI, previously suggested to be the result of an increased affinity for the drug-bound form of CCR5 (Pugach et al., 2007; Westby et al., 2007). As was proposed in these studies, when the affinity of the virus for inhibitor-bound CCR5 increases, the MI in drug susceptibility assays is reduced. Therefore, changes in the plateau height were proposed to be phenotypic markers of CCR5 co-receptor inhibitor resistance (Westby et al., 2007). However, in previous studies (Pugach et al., 2007; Westby et al., 2007), no mention of variations in plateau levels based on viral input was reported. This could be of significance since we found that plateaus in MI in single-cycle pseudovirus assays for the ADA-C2-$V5_{res}$ chimeric envelopes directly correlated with virus input. In addition, the plateau levels in MI displayed by the parental RU570 vicriviroc-resistant virus are also dependent on virus dose in single-cycle susceptibility assays (P. Buontempo and R. Ralston, unpublished observations). Therefore, this phenomenon is not unique to the ADA-C2-$V5_{res}$ chimeric envelope, or the pseudovirus assay. This is the first report of an entry inhibitor resistant virus that displays a dose dependant degree of resistance. Further analysis using carefully controlled assays and additional resistant envelopes are needed to determine if other resistant viruses display this phenomenon. In addition, more accurate assays that measure envelope affinity to drug-bound receptor may help to clarify the mechanism of dose-dependent resistance.

In summary, this approach utilizing chimeric envelope genes offers an alternative method to define resistance to co-receptor antagonists beyond cloning the full-length envelope which does not always result in functional pseudoviruses. This technique demonstrated that transferring a C2-V5 gp120 domain into a heterologous envelope by homologous recombination recapitulates resistance to a co-receptor antagonist in the chimeric envelope, therefore limiting the need for unique restriction sites or transferring envelope into shuttle vectors. This methodology may prove to be useful for monitoring the development of resistance to co-receptor antagonists in HIV-1 infected patients.

Materials and methods

Reagents

Vicriviroc was synthesized at Schering-Plough Research Institute, Kenilworth, NJ. The pSI expression vector was purchased from Promega Corp. (Madison, WI).

HIV-1 plasmids and HIV-1 primary isolates

The HIV-1 clade G RU570 primary isolate and the pNL4-3-AD8 molecular clone were obtained from the NIH AIDS Research and Reference Reagent program. The pNL4-$3E^{-}Luc^{+}$ and pSV7d-ADA gp160 plasmids were obtained from John Moore, Weill Medical College of Cornell University, New York, NY. The pcDNA3.1-RU570 gp160 clone (Parental) was constructed as follows. PBL cultures were infected with the RU570 primary isolate and an HIV DNA fragment corresponding to HXB2 nucleotide sequence 5739→8920 was PCR amplified from cell DNA using Platinum Pfx DNA polymerase (InVitrogen Corp., Carlsbad, CA) with the following primers:

Vpr-(F)-5′-ATAAGAATTCTGCAACAACTGCTG-3′
(HXB2: 5739–5762)
Nef-(R)-5′-CTCCATGTTTTTCTAGGTCTCGAGA-3′
(HXB2: 8896–8920)

The PCR product was gel purified and cloned into pCR®-Blunt II Topo vector (Invitrogen Corp.). RU570 gp160 was amplified from this construct using the following envelope specific primers:

Forward: 5′-CACCATGAGAGTGAAGGGGATACAGA-AG-3′
Reverse: 5′-CCTCCCATTTTATAGCAAAGCTCTTTC-3′

The 2.5 kb envelope PCR product was gel purified and cloned directly into pcDNA3.1, Version C vector (Invitrogen Corp).

Cell lines

The neoplastic T-cell line, PM-1, was obtained from the NIH AIDS Research and Reference Reagent program. PM-1 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS). U87 astroglioma cells expressing CD4 only, or with CCR1, CCR2, CCR3, CCR5, or CXCR4 were obtained from Dr. Dan Littman, New York University. Cells expressing CD4 only were maintained in DMEM supplemented with 10% FBS and 500 μg/ml G418 and cells expressing both CD4 and chemokine receptors were maintained in the same medium plus 1 μg/ml puromycin. 293T cells (CRL-11268) were purchased from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FBS and 300 μg/ml G418. PBL preparations from normal blood donors were obtained by leukophoresis performed at the New York Blood Center. PBLs from each donor were purified by centrifugation over a Ficoll-Hypaque density gradient (2000 rpm, 30 min). The cells were washed twice with PBS and pools from 6 to 8 donors were frozen in aliquots (5×10^7 cells per vial). Cells were cultured in RPMI supplemented with 10% FBS and 50 U/ml interleukin-2 (IL-2) and stimulated with 5 μg/ml phytohemagglutinin for 3 days prior to infection.

In vitro generation of RU570 virus cultures resistant to vicriviroc

The Hut 78 derived PM-1 (Lusso et al., 1995) T-cell line was infected with a parental PBL stock of HIV-1 RU570 for 1 week prior to the initiation of vicriviroc selection starting at 0.1 nM. PM-1 cell cultures were passaged weekly by transferring 1 ml of cell culture supernatant and one tenth of the infected cells into fresh PM-1 cultures ($1–2\times10^6$) in the absence (passage control

culture, RU570-PC) or presence (RU570-VCV) of vicriviroc. Culture supernatants were subsequently used to re-infect fresh PM-1 cells weekly. Viral supernatants and infected cells were collected periodically for p24 antigen titers, vicriviroc sensitivity testing in PBL described below in the viral replication assay section, and sequence analysis. RU570 virus initially grew poorly in the presence of vicriviroc, hence the concentration of vicriviroc was held below 1 nM for the first 12 weeks. As the replication of RU570 in the presence of vicriviroc increased, the concentration of vicriviroc was slowly increased to 400 nM between weeks 12 and 22 and this concentration was held constant until week 36. To select for minor populations of highly resistant variants that may have emerged, the concentration of vicriviroc was escalated to 10 μM at week 36 and cultures were maintained in 10 μM vicriviroc until viral replication remained consistent. By approximately week 56, VCV-treated cultures were replicating to similar levels compared to the passage control cultures and maintained consistently robust growth up to 93 weeks when cultures were terminated.

Viral replication assays

IL-2/PHA-stimulated PBL were seeded into 96-well plates (200,000 cells/well) pretreated with an equal volume of medium plus vicriviroc or medium only at 37 °C for 1 h. The plates were centrifuged at 300×*g* for 10 min and the media aspirated and replaced with 20 μl of fresh medium with or without compound. The cells were then infected in triplicate using 20 μl of viral supernatant (RU570) from 3hr to overnight at 37 °C. The cells were washed twice with PBS to remove residual viral inoculums and cultured in the presence or absence of vicriviroc for 4 to 6 days. Viral replication was assessed by measuring p24 antigen production by ELISA. The IC_{50} and IC_{90} values for vicriviroc susceptibility with these viruses were determined by analyzing dose–response curves using a 4-parameter logistic curve fit model with Prism GraphPad Software Version 4.0 (San Diego, CA).

Genotypic analysis of HIV-1 gp120 sequences

U87-CD4-CCR5 cells were infected with RU570 passage control or RU570 vicriviroc-resistant viruses collected following 93 weeks in culture. Forty-eight hours post infection total cellular DNA was isolated using Qiagen DNeasy tissue kit (Valencia, CA) and HIV-1 gp120 sequences were amplified by DNA-PCR from genomic DNA using the following restriction-site designed primers: *Sfi*I-gp120 1F 5′-*GCGGCCCAGCC-GGCC*AVAGTGAWGGRGAWACAGARGAATTGG-3′ corresponding to the HXB2 genome nucleotide sequence 6228–6255 and *Xho*I-gp120 1R 5′-*GGCTCGAGA*TCTTTTTTCTCT-CYSCACCACTCTTCTCY-3′ corresponding to the HXB2 genome nucleotide sequence 7729–7757. Special nucleotide designators are as follows: V = A/C/G, W = A/T, R = A/G, Y = C/T. HIV-1 gp120 sequences were amplified with Roche Expand High Fidelity PCR system (Roche Applied Science, Mannheim, Germany) using the following 30 cycle elongation program: 94 °C, 30 s, 55 °C, 45 s, and 68 °C, 4 min. PCR products were digested with *Sfi*I and *Xho*I restriction endonucleases (New England Biolabs, Beverly, MA), gel purified on 1% agarose and cloned directly into pSECTag2 (Hygro A) vector (InVitrogen Corp., Carlsbad, CA). DNA sequence analysis of individual clones was performed using a CEQ 2000 Dye terminator cycle sequencer (Beckman-Coulter Inc., Fullerton, CA).

Homologous recombination of HIV-1 gp120 fragments into pSV7d-ADAgp160 and pSI-RU570 PC gp160 expression vectors

The pSI-RU570 PC gp160 expression vector was constructed following PCR amplification of gp160 using restriction-site designed primers: *Mlu*I-Env4F 5′-*GGGACGCGT***ATG**AVAGT-GAWGGRGAW-3′ and *Xba*I-Env4R 5′-AAA*TCTAGA*TTT-GACMAYTTGCCHCCCATYTTA-3′ corresponding to envelope sequences flanking the start and stop codons of HIV-1 gp160, respectively. Special nucleotide designators are M = A/C and H = A/C/T. HIV-1 gp160 sequences were amplified with Roche Expand High Fidelity PCR system (Roche Applied Science, Mannheim, Germany) using the following 30 cycle elongation program: 94 °C, 30 s, 55 °C, 45 s, and 68 °C, 7 min. PCR products were digested with *Mlu*I and *Xba*I restriction endonucleases (New England Biolabs, Beverly, MA), gel purified on 1% agarose, and cloned directly into the pSI expression vector.

Homologous recombination of RU570 vicriviroc-resistant gp120 (RU570-VCV_{res}) into the pSI-RU570 passage control gp160 (RU570-PC) expression vector was performed as follows. The RU570-VCV_{res} gp120 clone #8 in pSECTag2 was digested with *Ale*I and *Mfe*I restriction endonucleases and the 730-bp C2-C5 gp120 fragment (HXB2 gp120 nucleotides 681–1432; gp120 aa position 228–478) was gel-purified on 1.0% agarose. The pSI-RU570-PC expression vector was digested with *Bsr*GI and *Sbf*I restriction endonucleases, followed by alkaline phosphatase treatment. The 5.8 kb vector fragment was gel purified on 1% agarose. Homologous recombination was performed using a 10-fold molar excess of gp120 fragment to vector for transformation of chemically competent *BJ5183 E. coli*, and individual colonies were screened by PCR for successful recombination.

Homologous recombination of the V2-C5 gp120 fragment from RU570-PC gp120 clone #16 and RU570-VCV_{res} gp120 clone #8 was performed following restriction endonuclease digestion of constructs with *Mfe*I and gel purification of the 838 bp V2-C5 gp120 fragments (HXB2 gp120 nucleotides 533–1431; gp120 aa position 185–478). The pSV7d-ADA gp160 expression vector was digested with *Bgl*II and *Bst*Z17I restriction endonucleases, alkaline phosphatase treated, and the 5.1 kb vector was gel purified on 1% agarose. A 50:1 ratio of V2-$C5_{res}$ gp120 fragment to vector was used to transform chemically competent *BJ5183 E. coli*, and individual colonies were screened by PCR for successful recombination.

Generation and characterization of HIV-1 pseudoviruses

HIV-1 pseudoviruses were produced in 293T cells by calcium phosphate transfection of pNL4-$3E^{-}Luc^{+}$ and HIV-1 envelope expression vectors using ProFection® Mammalian Transfection

system (Promega Corp., Madison, WI). HIV-1 pseudovirus was harvested in culture supernatants 48 h post-transfection and supernatants were clarified of cell debris by centrifugation at 1500×*g* for 10 min. Single-cycle infection assays were generally performed on the same day HIV-1 pseudovirus was harvested. To assess susceptibility to CCR5 co-receptor antagonists, 5000 U87-CD4-CCR5 cells/well were seeded into 96-well luminometer plates (Perkin Elmer) and plates were incubated overnight at 37 °C. The next day, serial 10-fold dilutions of inhibitor in cell culture medium (10 μM→1.0 pM) were added to wells 1 h prior to the addition of HIV-1 pseudovirus plus inhibitor. Plates were incubated for 72 h, and luciferase activity was analyzed by adding 50 μl of BrightGlo™ luciferase assay buffer and plates were read on a Dynex luminometer (300 mSec/well). Relative light units (RLU) were normalized to virus dose, measured as ng p24, and percent inhibition was calculated as follows: 100 − [average normalized RLU for HIV-1 pseudovirus plus drug / average normalized RLU for HIV-1 pseudovirus from control wells without drug] × 100. Dose–response data were analyzed using a nonlinear regression 4-parameter logistic curve fit program with Prism GraphPad Software Version 4.0 (San Diego, CA).

Site-directed mutagenesis (SDM) of gp120 amino acids

SDM of individual, gp120 amino acids in the pADA-C2-$V5_{res}$ clone was performed using QuikChange SDM kit (Stratagene, La Jolla, CA). Amino acid changes corresponding to HXB2 gp120 amino acid coordinates are as follows: R305K, Q315R, T319K, P363S, A373T, N413T, S437P, and T467I. All sequence changes were verified by DNA sequence analysis.

HIV-1 molecular clones

The pNL4-3-AD8 molecular clone was modified by deleting 650 bp of cDNA sequence flanking the 5′LTR, thereby creating a unique *Stu*I site within gp120. The ADA-$V3_{res}$ and ADA-C2-$V5_{res}$ chimeric envelopes were digested with *Stu*I and *Ale*I restriction endonucleases and the 1.2 kb env fragments corresponding to aa 203–608 (HXB2 gp160 aa coordinates) were gel purified on 1% agarose. These fragments were cloned directly into pNL4-3-AD8 digested with the same restriction endonucleases and all constructs were verified by DNA sequence analysis.

Generation of replication-competent HIV-1 viruses

293T cells were transfected with pNL4-3-AD8, pNL4-3-AD8-C2-$V5_{res}$, and pNL4-3-AD8-$V3_{res}$ molecular clones using Superfect transfection reagent (Qiagen Corp., Vencia, CA) and HIV-1 virus was harvested in supernatants 48 hr post transfection.

Nucleotide sequence accession numbers

The following HIV-1 envelope sequences have been submitted to GenBank: 2 sequences for RU570 gp160 (accession no. EU090200–EU090201) and 22 gp120 sequences for RU570-derived viruses (accession no. EU090202–EU090223).

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